Sympatric metabolic diversification of experimentally evolved Escherichia coli in a complex environment

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Sympatric metabolic diversification of experimentally evolved *Escherichia coli* in a complex environment

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Abstract Sympatric diversification in bacteria has been found to contravene initial evolutionary theories affirming the selection of the fittest type by competition for the same resource. Studies in unstructured (well-mixed) environments have discovered divergence of an ancestor strain into genomically and phenotypically divergent types growing both on single and mixed energy sources. This study addresses the metabolic diversification in an *Escherichia coli* population that evolved over ~1,000 generations under aerobic conditions in the nutritional complexity offered by Luria–Bertani (LB) broth. The medium lacked glucose but contained a variety of other resources. Two distinct metabolically-diverged types, coinciding with colony morphologies, were found to dominate the populations. One type was an avid carbohydrate consumer, which could quickly utilize the available (alternative) substrates feeding into glycolysis. The second type was a slow grower, which was able to specifically consume acetate. The capacity to utilize acetate might be providing an advantage to this second type, suggesting an increased capability to deal with adverse conditions that occur in the later stages of growth. The diverged metabolic preferences of the two forms suggested differential and interactive ecological roles within the population. We postulate that these types used different alternative metabolic strategies occupying different niches in a sympatric manner as an outcome of adaptation to the complex environment.

Keywords Experimental evolution · Adaptive diversification · Metabolism · *Escherichia coli*

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Introduction

Recent experimental evolution studies have placed a focus on understanding the diversification of bacteria occurring in sympatry. Such studies have found a direct relationship between the extent of diversification and environmental heterogeneity. Rainey and Travisano (1998) first observed rapid diversification occurring in structured conditions in *Pseudomonas fluorescens* growing in multiple-substrate medium. They concluded that spatial structure, triggering an uneven distribution of resources, was required to induce observable diversification (Rainey and Travisano 1998). Other studies using spatially-structured environments also demonstrated diversification processes in other bacteria (Korona 1996; Ponciano et al. 2009; Reboud and Bell 1997). On the other hand, in unstructured (well-mixed) environments, the "competitive exclusion" principle is thought to place limits on the diversity of the population in the system, as

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competition for the same resource would select for just one (fittest) variant (Hardin 1960). However, contradictory evidence on this principle was recently produced in bacteria growing under sympatric conditions with just one limiting resource (Kinnersley et al. 2009). The majority of these studies using one limiting substrate provided evidence that although there is a lack of structure, this one is later created from the interactions among new forms and their metabolic activities. Furthermore, the outcome of selection in unstructured conditions was re-examined using a mixture of energy sources. MacLean et al. (2005) found metabolic differentiation of different genotypes of *Pseudomonas* growing sympatrically and competing for the mix resources during 24 days of cultivation (MacLean et al. 2005). Moreover, several studies confirmed the diversification of *Escherichia coli* growing in mixed resources (Friesen et al. 2004; Hall and Colegrave 2007; Le Gac et al. 2008; Puentes-Téllez et al. 2013, 2014). Hence, media containing mixes of resources can be seen to provide a form of "structure" under which an organism may differentially specialize (Kassen et al. 2002, 2004).

Diversification may thus occur in an environment with multiple resources as a consequence of intense competition for commonly-used energy sources, yielding specialized forms that shift their nutritional preference and are better able to use as-yet-unexploited resources (MacLean et al. 2005). New niches may thus emerge through the actions of the organisms themselves. In such instances, diversity is created which can lead to coexistence maintained through interactions between the different types that are present.

Early work has already revealed particular emerged diversities on the basis of the rates of uptake of specific substrates (Claassen and Kortstee 1986; Helling et al. 1987), patterns of gene expression (Kinnersley et al. 2009; Kurlandzka et al. 1991; Rosenzweig et al. 1994) and genetic changes (Adams et al. 1992; Herron and Doebeli 2013). However, analyses of diversification in sympatric conditions have been limited to the use of only glucose or mixtures of glucose with just a few energy sources (Helling et al. 1987; Friesen et al. 2004). It is felt that greater environmental and ecological complexity should be integrated into the studies to get closer to the complexity of natural and industrial settings (Dettman et al. 2012). In the current study, we included high nutritional complexity in the factor environment by utilizing a medium, Luria–Bertani (LB) broth, which contains a complex mixture of energy sources and lacks glucose (Hanko and Rohrer 2004). This is, to our knowledge, the first study reporting metabolic diversification obtained after evolution in this highly used and complex medium, which is composed of complex substrates commonly used in industrial fermentations. Thus, we studied diversification in an *E. coli* population that had evolved over ~1,000 generations under aerobic conditions and was previously shown to possess genomic and transcriptomic heterogeneities with enhanced fitness relative to the ancestor (Puentes-Téllez et al. 2013, 2014). The scope of this work includes the investigation of metabolic patterns emerging in such heterogeneous populations, next to the dynamics of diversification, using colony morphology and differential metabolic behavior as the criteria.

Materials and methods

Strains and classification based on colony morphology

We use an end-point population of an *E. coli* K12 MC1000 culture that had undergone selection in serial-batch growth under aerobic conditions in LB broth (Puentes-Téllez et al. 2013). The daily transfers (after 24 h) had exposed the population to alternating periods of 'feast and famine' (cycled short-exponential and a long-stationary phases). Daily propagation of stationary-phase cells (at concentration of $\sim 1 \times 10^9$ CFU/ml) had been performed by applying a 100-fold dilution into fresh medium. After 150 days of selection (~1,000 generations obtained) a sample of the end-population was spread onto three different LB agar plates to isolate colonies. After overnight growth at 37 °C, a total of 30 colonies were randomly selected from all the plates. These colonies were observed using a stereoscope (40x) and classified using colony size and morphology. Cells belonged to three morphological types (defined here as "forms"), denoted "a", "b" and "c". All colonies were then sampled to be used for PCR fingerprinting using ERIC PCR primers (ERIC-I: ATgTAAgCTCCTggggATTCAC; ERIC-II: AAgTAAgTgACTggggTgAgCg) (Versalovic et al. 1991) and run in an agarose gel to confirm identity relative to the ancestral strain.

Metabolic assessment

The metabolic responses of cells from all selected colonies were assessed using Biolog GN2 plates (Biolog, Hayward, CA, USA). To this end, a clone originating from each of the 30 colonies was obtained by re-isolation on LB agar. Cells were resuspended in inoculation fluid (IF) provided by Biolog to an optical density (OD, 590 nm) of 0.070 ± 0.005. The microplates were then inoculated and incubated at 37 °C for 72 h. Absorbance values (turbidity at OD_{590}) were obtained at 24, 48 and 72 h of incubation. Data were interpreted as utilization versus no utilization of each substrate using a cutoff optical density of 0.2 (Maharjan et al. 2007). Data were then analyzed by constructing a multidimensional scaling (MDS) plot as well as by principal components analysis (PCA) of substrates categorized by their nature (Primer 6.1.13) (Clarke and Gorley 2006). The metabolic information of the ancestor was included in the analyses using the same setup. In the PCA, compounds that could not be classified within the main metabolic groups were assigned as "other compounds".

Maximal growth rate in specific substrates

Using triplicates, the growth of each form in 0.3 % of specific carbon sources (galactose, glucose, fructose, glycerol, mannose, L-arabinose, trehalose and acetate (0.03 %)) and in selected amino acids (arginine (0.6 mM), proline (0.4 mM), lysine (0.4 mM)) was tested in 100 ml flasks containing 30 ml of M9 minimal medium ($MgSO_4$ (3 mM), $CaCl_2$ (0.12 mM), and 24 % of M9 minimal salts 5X (Na_2HPO_4 (3.39 %), KH_2PO_4 (0.15 %), NaCl (0.25 %), NH_4Cl (0.5 %),0.0002 % thiamine and 0.1 % of a trace elements mix (2.5 g/l EDTA; 1.5 g/l FeSO4; 0.025 g/l CoCl2; 0.025 g/l ZnSO4; 0.015 g/l MnCl2; 0.015 g/l NaMoO4; 0.01 g/l NiCl2; 0.02 g/l H3BO3; 0.005 g/l CuCl2) (Jiménez et al. 2013), at 37 °C, with shaking at 200 rpm during 5 days. Growth was measured every 4 h (CFU/ml) by plating dilution series of each sample onto LB agar. The ancestor was included in the analyses. The maximum growth rate (V_{Max}, (Vasi et al. 1994)) under each specific substrate was calculated as the log transformed CFU/ml value against time during exponential growth phase; three estimates of V_{Max} were obtained per form in each substrate. In addition, growth of each form in LB broth was recorded every hour (sixfold) during 24 h at 600 nm using a microplate reader set with shaking before each read and at 37 °C (VersaMax, Molecular Devices Corp). After 24 h of growth, a sample of each well was plated onto LB agar to obtain the average number of cells per type (CFU/ml).

Results and discussion

We assessed the metabolic capabilities of individual morphotypes in an *E. coli* K12 population that evolved for ~ 1,000 generations in serial-batch LB broth cultures under aerobic conditions. Thirty colonies of the end-population were randomly picked from three different LB agar plates with an averaged count of 2.9×10^9 CFU/mL. The colonies that were picked showed different morphologies that showed to be stable when re-isolated on LB agar. All colonies were classified in three forms, as follows: (1) small colonies with diameters <1 mm, pronounced center and irregular shape ("fried egg" shaped) ("a" form), (2) large/rough/irregular colonies with diameters >1 mm ("b" form) and (3) large/smooth/irregular colonies with diameters >1 mm ("c" form) (See Fig. 1 and Puentes-Téllez et al. 2013). In our selection, 56 % of the colonies were form "a", whereas 20 and 23 % were forms "b" and "c" respectively. All picked colonies revealed ERIC-PCR patterns which were indistinguishable from those of the ancestral *E. coli* type, confirming their identity as evolved forms from this ancestor and discarding possible contamination (Fig. 1).

In previous work, although a significant level of consistency in genomic and phenotypic changes was found among the forms of this population, colony morphology together with other properties was found to correspond to differential genomic as well as gene expression backgrounds. For example, genomically, form "a" showed a higher number of "non-consistent" (not found in all the forms) mutations compared to forms "b" and "c". The latter two forms had a specific mutation in the major regulatory gene *arcA*. Gene *arcA* is a regulator with effects on nearly 170 genes, and it is involved in oxygen transitions. It is normally inactive during aerobic growth, but represses *rpoS* when active. The effect of differential changes in a high-impact regulatory gene like *arcA* are suggestive of differential phenotypic behaviors and potential

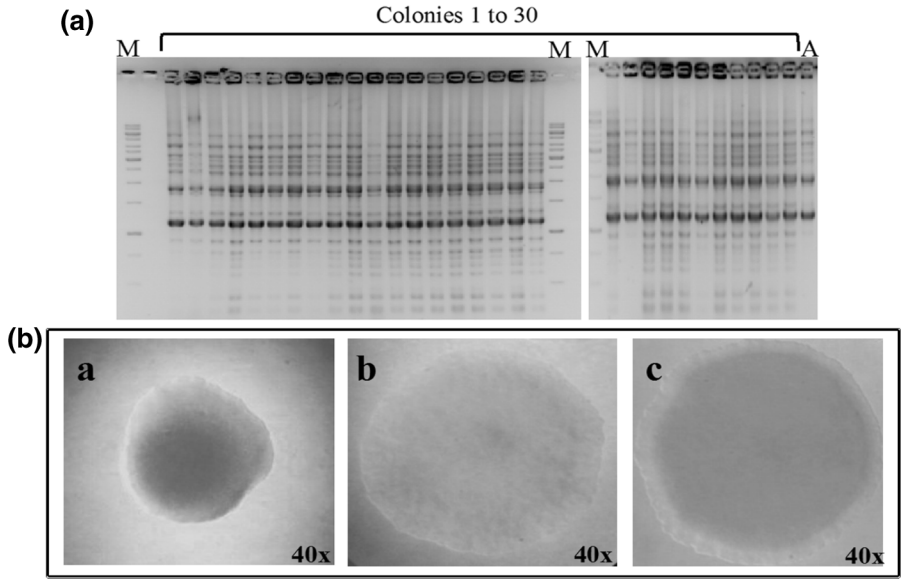

Fig. 1 **a** ERIC-PCR patterns of selected colonies (1–30) and the ancestor (A), M: 1 kb Marker. **b** Photography (40x) of the three observed morphologies (Forms "a", "b" and "c")

niche partitioning within the population. Moreover, higher numbers of genes were upregulated in forms "b" and "c" compared to form "a". However, form "a" showed a increased upregulation of key genes related to resistance to adverse conditions as well as those strongly related to colony morphology (e.g. *fim*) (Puentes-Téllez et al. 2013, 2014).

Diversity of metabolic patterns

Analyses of the metabolic capabilities of clones obtained after propagation of each of the 30 selected forms (and the ancestral type), were performed using Biolog GN2 plates. The collective data obtained after 72 h were used to construct a (normalized) multidimensional scaling (MDS) plot (Fig. 2). The analysis revealed that all forms, including the "a", "b" and "c" forms, clustered in two large groups, away from the ancestral type, on the basis of the degree of utilization of the 95 substrates. However, the ancestor appeared to be more similar to the metabolic "cloud" formed by form "a". Although there might be specific metabolic differences between forms "b" and "c", we clustered them together since they appear to have a fairly similar metabolic behavior. The analysis thus provided evidence for a presumed correlation between colony morphology and metabolic behavior, with forms "b" and "c" (larger and irregular colonies) being more similar and clearly different from form "a" (smaller-"fried-egg" shaped) and the ancestor. The results then clearly showed that two "metabolic types" were dominating the population.

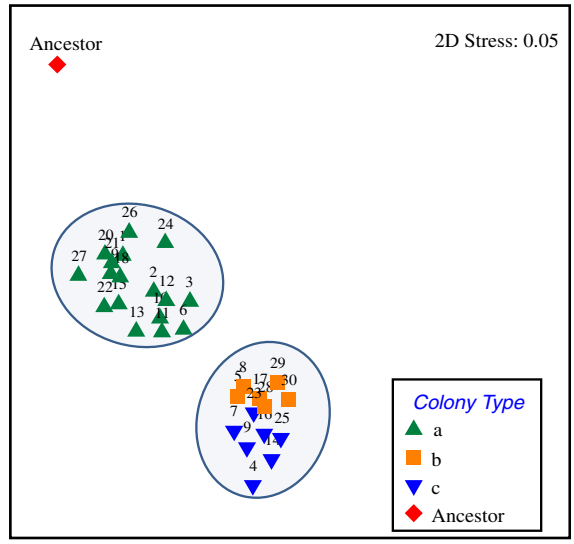

Fig. 2 MDS plot for square-root normalized Biolog results using Euclidean distance. Distance was obtained from the values obtained in an end-point measurement (after 72 h of incubation) of 30 randomly selected colonies. There are two main clusters suggesting two different metabolic patterns among the selected colonies

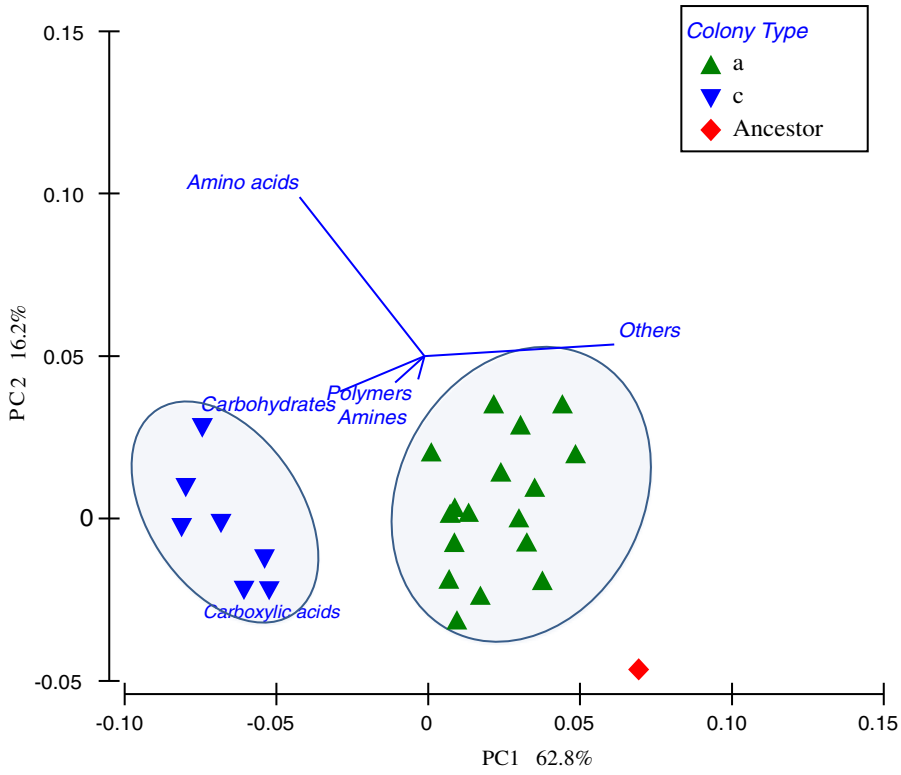

Fig. 3 Principal component analysis with Biolog substrates classified by their nature, using morphology types *a* and *c*. Samples cluster in two different groups. The first axis explains most of the variation (62.8 %), mainly triggered by carbohydrates, carboxylic acids amino acids and "other compounds" of the Biolog plate. The ancestor appears to have less metabolic distance to type *a*

We then used PCA to more closely observe the drivers of the Biolog substrate diversification. The analysis was performed using only the two observed "metabolic types", being metabolic type *a* (form "a") and the metabolic type *c* (form "c" used as a proxy for forms "b" and "c" together) (Fig. 3). The PCA revealed that most of the variation and metabolic distance between the two types is explained by the first axis (62.8 %). At a "substrate-type" level, this distance involved carbohydrates, carboxylic acids, amino acids and a group of "other (unclassified) compounds".

Metabolic preferences of the two selected metabolic types

Table 1 shows the average of the OD_{590} values obtained after 72 h in the Biolog plates for the most explanatory variables, listing only the substrates that showed significant differences ($p < 0.05$) between the two metabolic types. It also shows the response of the ancestor to these specific substrates. All values were normalized to the respective blank (cell suspension without substrate) per colony morphology. Clearly, type *c* appeared to be an optimal carbohydrate consumer than type *a* and the ancestor. After 72 h, the majority of carbohydrates had been consumed by this type to an $OD_{590} > 1.0$, whereas type *a* showed an overall lower response in the utilization of these carbohydrates. According to the absorbance values (Table 1), type *c* grew faster on glucose as well as on glucose-related sugars (coupling to glycolysis).

Thus, monosaccharides such as N-acetyl-D-glucosamine, L-arabinose, D-fructose, L-fucose, D-galactose, D-mannose, L-psicose and β-methyl-d-glucoside were rapidly consumed by type *c*, as well as disaccharides such as α-D-lactose, maltose, lactulose, D-melibiose, D-trehalose and the sugar alcohol D-mannitol. Although LB broth does not contain glucose, the ancestral capability to metabolize glucose was maintained in the two selected types (although at lower degree than the other tested carbohydrates).

The enhanced carbohydrate utilization by type *c* might be the result of a more active metabolic cascade in the central metabolism. This is consistent with the measured faster response by type *c* (more than 50 % faster, significant difference $p < 0.05$) when grown on carboxylic acids related to specific sugars

Table 1 Biolog Results. OD $_{590}$ values obtained for the most discriminative (significantly different) substrates between the two "metabolic types" *a* and *c* after 72 h of growth. Significant difference: two-tailed *t* test ($P < 0.05$). Absorbance values obtained by the ancestor growing in these substrates are displayed in order to show a statistical comparison with the closest type (type *a*). All values have been normalized as 000 = OD < 0.2

Substrates	Type *a*	± SEM	Type *c*	± SEM	Ancestor	± SEM
Carbohydrates						
N-acetyl-D-glucosamine	0.663	0.095	1.272	0.068	0.454	0.126
L-arabinose	0.433	0.042	1.130	0.088	0.000	0.000
D-fructose	0.403	0.042	1.041	0.081	0.341	0.093
L-fucose	0.893	0.055	1.256	0.141	0.266	0.041
D-galactose	0.588	0.027	1.350	0.148	0.000	0.000
D-glucose	0.350	0.045	0.920	0.099	0.479	0.045
α-D-lactose	0.505	0.070	1.248	0.066	0.000	0.000
Lactulose	0.985	0.055	1.404	0.072	0.000	0.000
Maltose	0.664	0.067	1.256	0.082	0.672	0.186
D-mannitol	0.866	0.081	1.307	0.142	0.543	0.083
D-mannose	0.513	0.054	1.145	0.098	0.431	0.153
D-melibiose	1.006	0.051	1.467	0.074	0.000	0.000
β-methyl-D-glucoside	0.357	0.039	0.885	0.080	0.000	0.000
D-psicose	0.000	0.000	0.219	0.011	0.000	0.000
L-rhamnose	1.244	0.062	1.520	0.038	0.281	0.000
D-sorbitol	1.024	0.070	1.485	0.028	0.381	0.016
D-trehalose	0.795	0.099	1.276	0.093	0.527	0.057
Carboxylic acids						
Pyruvic acid methyl ester	0.290	0.040	1.063	0.095	0.000	0.000
Acetic acid	0.463	0.017	0.000	0.000	0.000	0.000
D-galactonic acid lactone	0.677	0.047	1.490	0.125	0.389	0.157
D-galacturonic acid	0.281	0.047	1.428	0.081	0.376	0.239
D-gluconic acid	0.533	0.080	1.204	0.067	0.505	0.010
D-glucuronic acid	0.637	0.120	1.461	0.080	0.212	0.001
α-ketoglutaric acid	0.792	0.046	1.632	0.076	0.000	0.000
D,L-lactic acid	0.827	0.101	1.636	0.061	0.547	0.100
Propionic acid	0.538	0.034	0.680	0.022	0.000	0.000
D-saccharic acid	0.287	0.026	0.000	0.000	0.340	0.062
Succinic acid	0.425	0.046	1.084	0.076	0.000	0.000
Amino acids						
D-alanine	0.857	0.084	1.330	0.132	0.000	0.000
L-alanine	0.672	0.037	1.709	0.084	0.000	0.000
L-alanyl-glycine	0.439	0.041	1.600	0.110	0.201	0.058
L-asparagine	0.659	0.031	1.415	0.168	0.249	0.113
L-aspartic acid	0.596	0.068	1.871	0.091	0.233	0.096
L-glutamic acid	0.000	0.000	0.741	0.080	0.000	0.000
Glycyl-L-glutamic acid	0.000	0.000	0.304	0.046	0.000	0.000
L-proline	0.425	0.053	1.565	0.134	0.212	0.011
D-serine	0.446	0.067	1.404	0.035	0.343	0.065
L-serine	0.513	0.045	0.285	0.056	0.000	0.000

Table 1 continued

Substrates	Type *a*	± SEM	Type *c*	± SEM	Ancestor	± SEM
L-threonine	0.000	0.000	0.225	0.010	0.212	0.002
γ-aminobutyric acid	0.000	0.000	0.208	0.018	0.000	0.000
Amines						
Glucuronamide	0.435	0.057	1.113	0.130	0.000	0.000
L-alaninamide	0.000	0.000	0.221	0.018	0.000	0.000
Other compounds						
Bromosuccinic acid	0.387	0.021	0.514	0.046	0.000	0.000
Thymidine	0.898	0.039	1.169	0.067	0.293	0.071
Glycerol	0.770	0.022	1.520	0.048	0.232	0.022
D,L,α-glycerol phosphate	0.478	0.014	0.797	0.070	0.206	0.008
D-glucose-6-phosphate	0.251	0.011	1.017	0.064	0.366	0.154

In grey significantly different compared to type *a* ($p < 0.05$), *SEM* Standard error of the mean

(glucose, galactose and lactose) such as D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, D,L-lactic acid, next to three carboxylic acids included in the central metabolic pathway, i.e. pyruvic acid methyl ester, α-ketoglutaric acid and succinic acid (all three part of the tricarboxylic acid cycle (TCA cycle)) (Table 1).

Many glycolytic enzymes are constitutively expressed in *E. coli*, however those of the TCA cycle are inducible. Thus, the TCA cycle can respond differently to the presence of oxygen and carbon sources (Han, 2002). Hence, differences in the utilization of carbon sources between the two types (*a* and *c*) may have triggered the difference in response to the carboxylic acids of the TCA cycle.

We also found that the majority of amino acids were consumed faster by type *c* than by type *a* as well as the ancestor. *E. coli* can utilize amino acids as structural components next to their use as carbon, nitrogen or energy sources. Type *c* appeared to have evolved a better capacity to metabolize amino acids. In particular D- and L-alanine, D-alanyl-glycine, L-asparagine, L-aspartic acid, L-proline, L-threonine γ-aminobutyric acid, D and L-serine. These amino acids have been found to serve as excellent carbon and nitrogen sources (Chan and Newman 1981; Dover and Halpern 1972). L-glutamic acid and glycyl-L-glutamic acid were consumed to a lower extent by type *c*. Although glutamate is rarely used by *E. coli K12* as a carbon source, it is a common nitrogen source. Furthermore considerable amounts of glutamic acid have been found in tryptone and yeast extract and several mutants have been reported to grow on this amino acid as sole carbon source (Halpern and Umbarger 1961; Marcus and Halpern 1969).

Concerning the amine group, there was an enhanced utilization of glucuronamide and L-alaninamide by type *c*. In the group of "other" compounds, we found an enhanced utilization of glycerol—and its metabolically related compound D,L,α-glycerol phosphate—by type *c* (as compared to the ancestor and type *a*). *E. coli* growing aerobically on glycerol slots the compound into the central metabolism as dihydroxyacetone phosphate (DHAP), a metabolite which can participate in both gluconeogenic and glycolytic processes (Martínez-Gómez et al. 2012), and so one can presume that this pathway had an enhanced rate in type *c*. Although not having a strong impact on the separation of the types in the PCA graph (observed when excluded; data not shown), the "other compounds" group revealed other substrates showing differences between types *a* and *c* related to glucose metabolism (thymidine, D-glucose-6-phosphate), which is consistent with the premise of type *c* cells being adaptive in the sense of a more active metabolism of carbohydrates.

Type *a,* on the other hand, revealed a metabolic profile which resembled that of the ancestor. Thus,

Table 2 *V max* in specific substrates. *V Max* (h^{-1}) in M9 minimal medium with specific substrates as sole energy source

Substrate	a (h^{-1})	(± SEM)	c (h^{-1})	(±SEM)	Ancestor (h^{-1})	(±SEM)
Galactose 0.3 % †	0.052	0.004	0.191*	0.002	0.032	0.006
Glucose 0.3 %	0.004	0.002	0.162*	0.004	0.013	0.000
Fructose 0.3 %	0.015	0.002	0.133*	0.005	0.023	0.010
Glycerol 0.3 %	0.017	0.003	0.184*	0.002	0.021	0.004
Mannose 0.3 %	0.022	0.002	0.172*	0.003	0.010	0.001
Trehalose 0.3 %†	0.023	0.003	0.218*	0.016	0.034	0.004
L-arabinose (0.3 %)†	0.090	0.011	0.130	0.009	0.000	0.000
L-arginine 0.6 mM†	0.053	0.005	0.040	0.002	0.017	0.001
Lysine 0.4 mM†	0.013	0.001	0.011	0.001	0.016	0.008
Acetate 0.03 %	0.063*	0.003	0.032	0.004	0.022	0.008
Glycine 0.4 mM†	0.043	0.005	0.066	0.000	0.081	0.011
Proline 0.4 mM†	0.030	0.006	0.067*	0.002	0.010	0.002

† Substrates present in LB broth (Hanko and Rohrer 2004; Baev et al. 2006)

* Significant difference calculated between types *a* and *c*. $p < 0{,}05$ (two-tailed *t* test) *SEM* standard error of the mean

there were similar differences between types *c* and *a* as between type *c* and the ancestor. However, some differences between type *a* and the ancestor were found to be significant (Table 1, in grey, $p < 0.05$). Whereas type *a* was able to utilize several sugars and amino acids (even to a low degree), the ancestor did not utilize these or did so to a much lower degree. Thus, generally speaking, the ancestor revealed a more restricted metabolic potential than the evolved types.

Maximal growth rate in specific (LB related) substrates

To analyze the response to specific substrates of LB broth, we performed growth experiments with types *a* and *c*. LB lacks glucose (Hanko and Rohrer 2000, 2004) and the two major components of LB, tryptone and yeast extract (YE), are derived from complex biological sources. These two components typically contain partially digested proteins (tryptone), vitamins (YE), next to a variety of trace carbohydrates and unknown substances (Hanko and Rohrer 2004). Both components thus contain 7.7 and 17.5 % of carbohydrates, respectively (BD, Franklin Lakes, New Jersey), at a final carbohydrate concentration of about 0.16 %. Previous studies confirmed the presence in LB of considerable amounts of trehalose, D-galactose, L-arabinose, D-mannose, melibiose and L-fucose, among other compounds (Baev et al. 2006; Hanko and Rohrer 2000). However, in *E. coli* K-12 MG1655 cultures, steady-state growth ceased early (at an OD_{600} of approx. 0.3). This shift occurred in particular when the available carbohydrates were consumed, and the ensuing lowering of growth rate forced cell metabolism to switch to the use of amino acids as carbon source (Sezonov et al. 2007). The metabolic differences between types *a* and *c* can be thought of as indicating niche differentiation and (differential) evolutionary adaptation of these two types to the environment. We included various substrates in minimal medium in order to shed light on the different metabolic routes taken by types *a* and *c* (Table 2). Samples were taken at 4-h intervals, given the slow growth in the minimal medium, with just one substrate supporting growth as a carbon and energy source. Thus, maximal growth rates were determined (V_{Max}) using substrate concentrations that were convenient for estimating V_{Max}, i.e. the limiting maximal growth rate that is adopted by the microbial growth (Vasi et al. 1994).

The results (Table 2), confirmed the faster growth of type *c* than of type *a* as well as the ancestor on all tested carbohydrates ($p < 0.05$). In contrast, the response to amino acids was not significantly different (L-arginine and lysine offer both carbon and nitrogen sources to *E. coli*), with the exception of type *c* growing with L-proline (0.4 mM). L-proline is well utilized by many *E. coli* strains as an excellent carbon/nitrogen and energy source (Chen and Maloy 1991). In general, *E. coli* apparently has evolved to handle mixtures of amino acids and complex oligopeptides, rather than single amino acids, as energy sources (Chen and Maloy 1991). Therefore, our analysis was expected to not reveal a clear picture of the metabolic capabilities of *E. coli* consuming this type of compounds.

Overall, type *c* exhibited a more active metabolism on available substrates, possibly indicating a higher metabolic flux, than type *a* as well as the ancestor. One of the factors that may most strongly limit the growth rate is the import of substrates into the cell ("metabolic bottleneck") (Chen and Maloy 1991). A highly active carbohydrate metabolism is often controlled by major regulatory systems like the carbohydrate phosphotransferase system (PTS), which catalyzes the transport/uptake of carbohydrates and their coupling to glycolysis. This PTS has been found to assure the optimal utilization of carbohydrates in complex environments (Kotrba et al. 2001). Since glucose is absent from LB broth, the type *c* cells may have developed a strategy based on alternative ways to get substrate for the glycolysis pathway and the central burning of carbon.

Interestingly, type *a* showed a significantly different response from that of type *c* when growing in minimal medium with acetate as the sole carbon and energy source, confirming the higher utilization of acetate that was already observed in the Biolog plates (Table 1). Acetic acid is a (released) by-product of the rapid aerobic growth of *E. coli* (Han 1992). Its release is particularly associated with high rates of metabolism of glucose or glucose-related compounds. Acetate may actually establish adverse conditions to fast-growing cells. Production of acetate can be significant in batch fermentations, given the extended growth phase in such systems, which allows this compound to attain elevated concentrations. A metabolic switch has been observed when such adverse conditions occur in an environment in which oxygen and glucose decrease by 50 to 80 %, with a concomitant initiation of acetate utilization (Kleman and Strohl 1994). In this scenario, acetate can rapidly accumulate and become an alternative carbon source, allowing the evolution of divergent types that are able to exploit the new niche, maintaining interactions with other members of the system. These interactions may sustain diversification. Helling et al. (1987) and Rosenzweig et al. (1994) already demonstrated the presence of stable heterogeneity involving acetate in defined medium cultures that had originated from a unique ancestor (Helling et al. 1987; Rosenzweig et al. 1994). In the system, the use of glucose as the carbon and limiting energy source effectively increased and, as a consequence, overproduction of acetate ensued. Then, a type able to specifically utilize acetate emerged. Thus, the catabolic pathways of glucose, acetate and glycerol may be interconnected in a multiple-type population, counteracting the emergence of a single clone that consumes these substrates together. Instead, three metabolically different types were successfully maintained in the population. Although a clear limiting substrate was not present in our experimental setup, we found here that primarily type *c* may have used alternative pathways for the available resources in LB, feeding into glycolysis. Hence, the type *c* cells may have efficiently fed the central metabolic pathway, producing secondary metabolites like acetate and thus creating an available niche for the newly emerged type *a* which was able to utilize this substrate. The enhanced assimilation of acetate might be linked to an enhancement of acetyl-CoA synthetase (produced by the *acs* gene) (Lin et al. 2006); further studies will address this issue at the transcriptomic level in types *a* and *c*.

Dynamics of growth in LB broth

To observe the population dynamics of types *a* and *c* in the complete mosaic of resources offered by LB, growth experiments were performed for each type in separate in LB. Specific growth rates (μ) were determined by using the slope of the logarithm of growth in the exponential phase. Fig. 4 shows data collected over 24 h in 96-well microplates containing 100 uL of LB broth. The values of μ after 7 h were 0.22 h^{-1} (SD + 0.019) for type *a* and 0.26 h^{-1} (SD + 0.004) for type *c*. However, at the beginning of stationary phase (5 h later), when growth rates had decreased for both strains, type *c* had lowered its growth rate to 0.11 h^{-1} (SD + 0.002) compared to 0.17 h^{-1} (SD + 0.004) for type *a*. Thus, by the end of the measurement, there was a significant difference in the end-populations between types *a* and *c* ($p < 0.001$). Viable (CFU) counts confirmed this, with CFU/ml end-values being 1×10^9 CFU/ml for type *a* and 3.5×10^8 CFU/ml for type *c*. These results suggest a faster exponential growth of type *c* over type *a* as a result of the faster consumption of the available primary resources. On the other hand, type *c* rapidly reduced its growth rate upon depletion of the available sources. Apparently, type *a* was not a fast "primary source" eater, however it finished its growth later, with a higher final cell count than type *c*.

Friesen et al. (2004) revealed the presence of stable heterogeneity in *E. coli* lines that evolved in mixtures of glucose and acetate. Diauxic behaviour (sequential

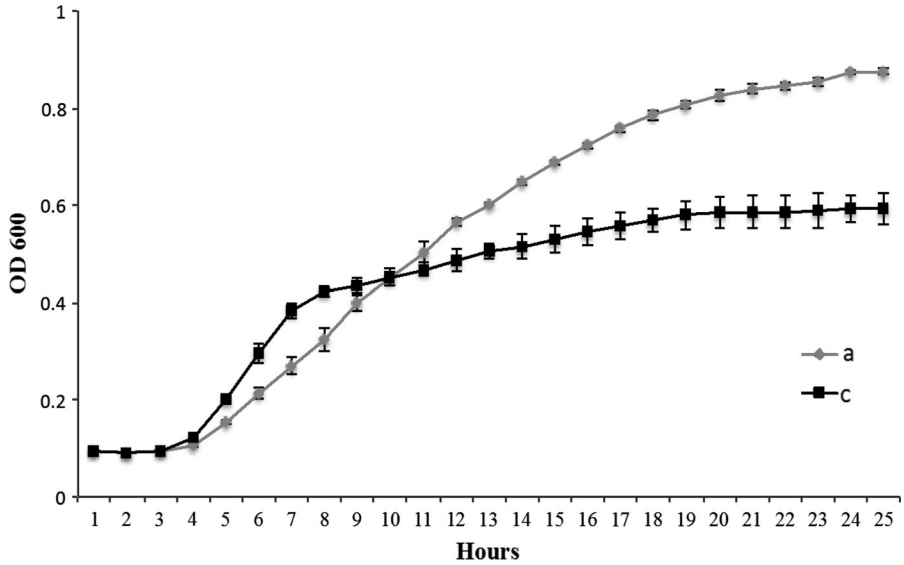

Fig. 4 Optical density (OD_{600}) of types *a* and *c* growing separately in LB broth during 24 h

metabolism of two energy sources resulting in a metabolic shift between these two substrates, producing two separate growth phases), in which the initial growth phase is correlated with the use of the preferred metabolite (e.g. glucose) and a subsequent growth phase with the use of the less preferred metabolite (e.g. acetate) was demonstrated (Tyerman et al. 2005).

Our results obtained in LB broth indicate a diauxic shift and the emergence of new niches which were occupied by the evolved types in an environment without a defined limiting substrate and with cycling periods of "feast and famine". Although the scope of this work was limited to the description of the dynamics of the diversification and not the mechanisms maintaining it, the results suggest the presence of forces like frequency-dependent selection acting upon these types under an environment of "feast and famine" with a multi-resource background. Although sympatric diversification has been demonstrated before in experiments with a defined mixture of just a few substrates (Friesen et al. 2004), showing splits of sympatrically growing linages at the genetic level (Herron and Doebeli 2013), the current study is—to our knowledge—the first one demonstrating such diversification in the presence of multiple substrates offered by LB without a pre-established limiting substrate. The metabolic behavior that we found in type *a* correlated with a slower use of the available mosaic of resources of LB. Such a lower growth rate in the early growth stages may offer an opportunity (to a then lower number of cells) to easily consume the available resources, and since generation time is larger, the population can make use of the resources for a longer time. In the light of its acetate consumption, type *a* also was a superior by-product consumer. These combined characteristics, i.e. a lower growth rate (often linked to enhanced stress resistance (Notley and Ferenci 1996)) next to an enhanced utilization of the by-product acetate, may confer on type *a* a unique survival capacity when together with type *c* under conditions that may be adverse to any other type. A future study will analyze the dynamics of the different metabolic types when growing together in LB and the presence of other "social" advantages between the members of the population.

Taken together, the results of this study confirm the emergence of heterogeneity and niche differentiation in an *E. coli* K12 MC1000 population after its evolution in serial batch cultures over ~1,000 generations. The habitat this population grew in represents a highly complex environment with a vast mixture of substrates providing a form of "structure" at a microscale. As a general rule for any habitat, the number of niches available for microbial niche occupants is thought to match the extent of environmental variation. Hence, given the presumed complexity in our habitat, even more subtle polymorphisms and other possible interactions might be assumed to occur. Here, we detected the clear emergence of two dominating metabolically-different types in the population, denoted "types" *a* and

c, (metabolic type *c* represented two forms, denoted forms "b" and "c"). Types *a* and *c* both developed novel alternative strategies to deal with the substrates in LB broth, which lacks glucose. Type *c* was a fast consumer of carbohydrates alternative to glucose, with a metabolic slot into the glycolysis pathway, whereas type *a* was a slow grower on such carbohydrates. However, given the ability of type *a* to consume acetate, it might be able to better cope with an environment that is created in a later stage of development, when primary resources have been consumed and conditions have become rather adverse. Since acetate is produced by high-rate glucose transformation pathways (in this case offered by alternative carbohydrates present in the media) (Dittrich et al. 2005), type *a* might profit from the production of this by-product, reincorporating it into its metabolism. The presence of interactions in the population correlates with the stable dynamic behavior in the system. This study demonstrates an outcome of adaptation to complex environments commonly found in nature or in industrial settings involving the presence of multiple energy sources and the lack of a limiting substrate.

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Conflict of interest The authors declare that they have no conflict of interest.

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